Thromboxane A_2-induced contraction of rat caudal arterial smooth muscle involves activation of Ca^{2+} entry and Ca^{2+} sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697

David P. WILSON, Marija SUSNJAR, Enikő KISS, Cindy SUTHERLAND and Michael P. WALSH[1]

Smooth Muscle Research Group and Department of Biochemistry & Molecular Biology, University of Calgary Faculty of Medicine, 3330 Hospital Drive N.W., Calgary, Alberta, Canada T2N 4N1

The signal transduction pathway whereby the TxA_2 (thromboxane A_2) mimetic U-46619 activates vascular smooth muscle contraction was investigated in de-endothelialized rat caudal artery. U-46619-evoked contraction was inhibited by the TP receptor (TxA_2 receptor) antagonist SQ-29548, the ROK (Rho-associated kinase) inhibitors Y-27632 and H-1152, the MLCK (myosin light-chain kinase) inhibitors ML-7, ML-9 and wortmannin, the voltage-gated Ca^{2+}-channel blocker nicardipine, and removal of extracellular Ca^{2+}; the protein kinase C inhibitor GF109203x had no effect. U-46619 elicited Ca^{2+} sensitization in α-toxin-permeabilized tissue. U-46619 induced activation of the small GTPase RhoA, consistent with the involvement of ROK. Two downstream targets of ROK were investigated: CPI-17 [protein kinase C-potentiated inhibitory protein for PP1 (protein phosphatase type 1) of 17 kDa], a myosin light-chain phosphatase inhibitor, was not phosphorylated at the functional site (Thr-38); phosphorylation of MYPT1 (myosin-targeting subunit of myosin light-chain phosphatase) was significantly increased at Thr-855, but not Thr-697. U-46619-evoked contraction correlated with phosphorylation of the 20 kDa light chains of myosin. We conclude that: (i) U-46619 induces contraction via activation of the Ca^{2+}/calmodulin/MLCK pathway and of the RhoA/ROK pathway; (ii) Thr-855 of MYPT1 is phosphorylated by ROK at rest and in response to U-46619 stimulation; (iii) Thr-697 of MYPT1 is phosphorylated by a kinase other than ROK under resting conditions, and is not increased in response to U-46619 treatment; and (iv) neither ROK nor protein kinase C phosphorylates CPI-17 in this vascular smooth muscle in response to U-46619.

Key words: Ca^{2+} sensitization, L-type Ca^{2+} current, myosin light-chain phosphatase, thromboxane A_2, CPI-17, vascular smooth muscle.

INTRODUCTION

Thromboxane A_2 (TxA_2) is an unstable prostanoid metabolite of arachidonic acid produced predominantly in platelets, in response to a variety of physiological and pathological stimuli, through isomerization of prostaglandin H_2 by TxA_2 synthase [1,2]. TxA_2 induces smooth-muscle contraction and proliferation, platelet shape change and aggregation [1,3,4]. Its actions are mediated by the TP receptor (TxA_2 receptor), a G-protein-coupled receptor, which typically triggers activation of phospholipase Cβ via $G_{q/11}$, leading to an increase in $[Ca^{2+}]_i$ (cytosolic free Ca^{2+} concentration) and Ca^{2+} sensitization of contraction [5–8].

Since TxA_2 has been implicated in cardiovascular, renal and respiratory diseases [9], it is important to understand the signal transduction pathways underlying its mechanisms of action. To this end, the stable TxA_2 analogue U-46619 is commonly used, since TxA_2 itself is unstable. Interestingly, Himpens et al. [5] observed that TxA_2 elicited a modest increase in $[Ca^{2+}]_i$, but a large increase in force in rabbit pulmonary arterial smooth muscle, implying a robust Ca^{2+}-sensitizing effect of the prostanoid. This phenomenon of Ca^{2+} sensitization, whereby certain agonists elicit a contractile response with little or no rise in $[Ca^{2+}]_i$ [10], has been extensively studied in recent years. Two principal molecular mechanisms have emerged to explain Ca^{2+} sensitization, both involving inhibition of MLCP (myosin light-chain phosphatase) [11]. First, agonist-induced activation of the small GTPase RhoA via the $G_{12/13}$ family of heterotrimeric G-proteins and a GEF (guanine nucleotide-exchange factor) [12] leads to activation of ROK (Rho-associated kinase) [13], which phosphorylates MYPT1 (the myosin-targeting subunit of MLCP) at Thr-697 and Thr-855 (numbering used throughout is from the rat isoform; NCBI accession number Q10728) [14,15]. Secondly, activation of PKC (protein kinase C) via phospholipase Cβ-mediated production of diacylglycerol leads to phosphorylation of CPI-17 [protein kinase C-potentiated inhibitory protein for PP1 (protein phosphatase type 1) of 17 kDa], a cytosolic protein that becomes a potent MLCP inhibitor when phosphorylated at Thr-38 [16]. CPI-17 is also a substrate of ROK and other kinases [11], phosphorylation again occurring at regulatory Thr-38. Smooth-muscle contraction is activated primarily by phosphorylation of LC_{20} (20 kDa light chains of myosin II) by Ca^{2+}/calmodulin-dependent MLCK (myosin light-chain kinase) [17]. A decrease in MLCP activity will shift the balance in favour of MLCK, resulting in a greater degree of LC_{20} phosphorylation and contraction. Direct analysis of MYPT1 and CPI-17 phosphorylation in a variety of vascular smooth muscles suggests that their relative importance varies, depending on the agonist and tissue [18–23]. Although ROK has been shown to have the capacity to phosphorylate CPI-17, it is unclear whether this reaction occurs in the intact smooth-muscle cell.

Abbreviations used: Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CPI-17, protein kinase C-potentiated inhibitory protein for PP1 (protein phosphatase type 1) of 17 kDa; CPA, cyclopiazonic acid; DTT, dithiothreitol; GEF, guanine nucleotide-exchange factor; GST, glutathione S-transferase; GTP[S], guanosine 5′-[γ-thio]triphosphate; ILK, integrin-linked kinase; KLH, keyhole-limpet haemocyanin; LC_{20}, 20 kDa light chains of myosin II; M20, the 20 kDa subunit of myosin light-chain phosphatase; MLCK, myosin light-chain kinase; MLCP, myosin light-chain phosphatase; MYPT1, myosin-targeting subunit of MLCP; PdBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; cPKC, a mixture of the α, β and γ PKC isoenzymes; PP1c, the catalytic subunit of MLCP; ROK, Rho-associated kinase; SR, sarcoplasmic reticulum; TCA, trichloroacetic acid; TxA_2, thromboxane A_2; TP receptor, TxA_2 receptor; ZIP kinase, zipper-interacting protein kinase.

[1] To whom correspondence should be addressed (email walsh@ucalgary.ca).

Here, we describe the results of studies with intact and permeabilized vascular smooth muscle designed to identify components of the signalling pathway(s) involved in U-46619-induced contraction of rat caudal artery, an extensively studied vessel. More specifically, experiments were designed to determine whether ROK activation results in Ca^{2+} sensitization due to a change in the phosphorylation of CPI-17 at Thr-38, and of MYPT-1 at Thr-697 and Thr-855. We conclude that TxA_2, acting via TP receptors, triggers Ca^{2+} entry via L-type Ca^{2+} channels (leading to activation of MLCK and contraction) and Ca^{2+} sensitization, which involves activation of RhoA and ROK, but not PKC, and phosphorylation of MYPT1 at Thr-855, but not Thr-697.

EXPERIMENTAL

Materials

Reagents were purchased from the following sources: H-1152, GF109203x, wortmannin, di-isopropylfluorophosphate, cyclopiazonic acid, thapsigargin and A23187 were from Calbiochem-Novabiochem Corp. (San Diego, CA, U.S.A.); Y-27632 was from BioMol (Plymouth Meeting, PA, U.S.A.); SQ-29548 was from Cayman Chemical (Ann Arbor, MI, U.S.A.); Caps [3-(cyclohexylamino)propane-1-sulphonic acid], leupeptin, pepstatin A and [γ-^{32}P]ATP (> 5000 Ci/mmol) were from ICN Biomedicals Inc. (Aurora, OH, U.S.A.); ML-7 and ML-9 were from Toronto Research Chemicals Inc. (North York, ON, Canada); microcystin-LR was from Alexis Biochemicals (San Diego, CA, U.S.A.); Pefabloc SC® and GTP[S] (guanosine 5′-[γ-thio]triphosphate) were from Roche Applied Science (Laval, PQ, Canada); U-46619, benzamidine, Igepal CA-630, aprotinin, Mops and nicardipine were from Sigma–Aldrich Canada (Oakville, ON, Canada); L-α-phosphatidylserine and 1,2-diolein were from Doosan Serdary Research Laboratories (London, ON, Canada); Tween 20 was from Bio-Rad Laboratories (Mississauga, ON, Canada); and Tween 80 was from Fisher Scientific (Whitby, Ontario, Canada). All other chemicals (from VWR, Edmonton, AB, Canada or Sigma–Aldrich) were of analytical grade, or better. cPKC (a mixture of the α, β and γ isoenzymes) was purified as described previously [24]. Rabbit polyclonal antibodies specific for PP1c (the catalytic subunit of MLCP) and MYPT1 phosphorylated at Thr-697 (anti-[PThr697]-MYPT1) or Thr-855 (anti-[PThr855]-MYPT1) were purchased from Upstate USA, Inc. (Charlottesville, VA, U.S.A.). Anti-M20 (a polyclonal antibody against the 20 kDa subunit of MLCP) [25] was generously donated by Dr Greg Moorhead (Department of Biological Sciences, University of Calgary). A polyclonal phosphospecific antibody (rabbit IgG) that recognizes only CPI-17 phosphorylated at Thr-38 (anti-[PThr38]-CPI-17) [22] was generously provided by Dr Mitsuo Ikebe (University of Massachusetts, Worcester, MA, U.S.A.). Anti-CPI-17 (rabbit IgG) that recognizes both phosphorylated and unphosphorylated CPI-17 [19] was generously provided by Dr Masumi Eto (University of Virginia, Charlottesville, VA, U.S.A.).

Antibody production

To raise anti-[PThr697]-MYPT1-specific antibodies, two peptides were synthesized at the University of Calgary Peptide Synthesis Facility: MYPT1(691–703) [RQSRRST(P)QGVTLTC] with a C-terminal cysteine residue and phosphothreonine at the ROK phosphorylation site (Thr-697), and the corresponding unphosphorylated peptide. The phosphothreonine-containing peptide was coupled to KLH (keyhole-limpet haemocyanin) and injected into mice. Mice producing serum positive for MYPT1 binding were utilized for monoclonal antibody production. Anti-[PThr697]-MYPT1 was purified by affinity chromatography on a phosphopeptide column. Anti-MYPT1, which recognizes MYPT1 independently of phosphorylation status, was purified by affinity chromatography on a column containing the coupled unphosphorylated peptide. Polyclonal antibodies against RhoA were raised in rabbits by injection of a peptide (residues 173–190: MATRAALQARRGKKKSGC) coupled to KLH. The IgG fraction was purified from serum using High Trap Protein A (Amersham Pharmacia Biosciences, Baie d'Urfe, PQ, Canada). Anti-RhoA was purified further using a SulfoLink® affinity column to which the RhoA peptide was coupled via the C-terminal cysteine residue, following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, U.S.A.).

Purification of MLCP

Chicken gizzard (100 g) was minced, homogenized in a Waring blender (3 × 5 s) in 400 ml of 20 mM Tris/HCl, pH 7.5, containing 40 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT (dithiothreitol) and 0.05 % (v/v) Triton X-100, and centrifuged at 18 600 ***g*** for 15 min. The pellet was suspended in 400 ml of 20 mM Tris/HCl, pH 7.5, containing 40 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA and 1 mM DTT, homogenized and centrifuged as before. Suspension of the pellet and centrifugation were repeated. The resultant pellet was suspended in 400 ml of Mg buffer [20 mM Tris/HCl (pH 7.5)/60 mM NaCl/25 mM $MgCl_2$/1 mM EGTA/1 mM DTT], homogenized and centrifuged as before. The supernatant was filtered through glass wool and applied to a DEAE-Sephacel column (2.5 cm × 40 cm) equilibrated with Mg buffer. The column was washed with Mg buffer, and bound proteins were eluted with a linear [NaCl] gradient generated from 250 ml each of Mg buffer and Mg buffer containing 0.3 M NaCl. Fractions (4 ml) were collected at a flow rate of 45 ml/h and assayed for MLCP by Western blotting with anti-MYPT1. Fractions containing MYPT1 were pooled, purified further by microcystin–Sepharose affinity chromatography [26], dialysed and stored at − 80 °C. MLCP was determined to be > 95 % pure, and contained the expected subunits of approx. 130, 38 and 20 kDa, identified by Western blotting as MYPT1, PP1c and M20 respectively.

In vitro phosphorylation of MYPT1

To produce MYPT1 phosphorylated at Thr-697 and Thr-855, purified MLCP (0.3 μg) was incubated for 60 min at 30 °C with 2 μl of ROK (Upstate USA, Inc.) in kinase buffer [25 mM Tris/HCl (pH 7.5)/10 mM EGTA/50 mM KCl/10 mM $MgCl_2$/10 mM DTT/0.1 % Tween 80/20 μM microcystin-LR/0.2 mM PefaBloc SC®/1 mM benzamidine] in the absence or presence of 0.2 mM ATP. Reactions were stopped by addition of an equal volume of 2 × SDS gel sample buffer containing 10 mM EDTA, and boiling. Samples (15 μl) were loaded on gels for Western blotting.

Cloning of CPI-17 cDNA

mRNA was isolated from rat caudal arterial smooth-muscle tissue using reagents provided by Ambion (Austin, TX, U.S.A.). cDNA was prepared by RT (reverse transcriptase)-PCR from mRNA (77.5 ng) using an oligo$(dT)_{12–18}$ primer (Gibco–BRL, Carlsbad, CA, U.S.A.) and gene-specific primers based on the published rat CPI-17 sequence (NCBI accession number NP_569087) with engineered BamHI and EcoRI cut sites (underlined): forward primer, 5′-GAGAUGGATCCUGGCGTGATGGCAGCGCAGCGGCT-3′; and reverse primer, 5′-GAGAUGAATTCUCCGGTGGAGCAGTGTGAGCCGGGTC-3′. The 443 bp PCR product was electrophoresed, excised from a 1 % agarose gel and purified using a QIA quick gel extraction kit (Qiagen, Mississauga, ON,

Canada). The amplified CPI-17 was sequenced in the University of Calgary DNA Services Core Facility and found to be identical to the published rat sequence, with a few additional residues at the termini due to the engineered restriction sites (see below).

CPI-17 protein expression

CPI-17 cDNA subcloned into pGEX-2T (Amersham Pharmacia Biosciences) was expressed in *Escherichia coli* BL-21 (DE3) pLysS cells. The GST (glutathione S-transferase)–CPI-17 fusion protein was purified on a glutathione–Sepharose column (Amersham Pharmacia Biosciences), and the GST moiety was cleaved off with thrombin (1 unit/mg of protein). Two N-terminal amino acids (GV) were added from the vector, and five amino acids were added at the C-terminus (GIHRD). The thrombin-cleaved GST–CPI-17 was passed over another glutathione–Sepharose column to remove the GST moiety.

In vitro phosphorylation of CPI-17

Phosphorylation of recombinant CPI-17 (36 μg/ml) by cPKC (9.2 ng/ml) was carried out at 30 °C in 20 mM Tris/HCl, pH 7.5, containing 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.08 mg/ml L-α-phosphatidylserine, 0.008 mg/ml 1,2-diolein, 1 μM microcystin-LR and 0.2 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). Phosphorylation of recombinant CPI-17 (47.6 μg/ml) by PKCδ (0.5 μg/ml; Upstate USA, Inc.) was carried out at 30 °C in 7.6 mM Mops, pH 7.2, containing 0.4 mM DTT, 0.4 mM β-glycerophosphate, 0.05 mg/ml L-α-phosphatidylserine, 0.005 mg/ml 1,2-diolein and 0.2 mM [γ-^{32}P]ATP (200–500 c.p.m./pmol). Incorporation of [^{32}P]P_i into CPI-17 was quantified by Čerenkov counting, and was also assessed by SDS/PAGE and autoradiography [27].

Force measurements

Rat caudal arterial de-endothelialized smooth-muscle helical strips (6 × 1.5 mm) were prepared for force measurements, as described previously [28]. Mounted strips equilibrated in H-T solution [135.5 mM NaCl/5.9 mM KCl/1.2 mM $MgCl_2$/2.5 mM $CaCl_2$/11.6 mM Hepes (pH 7.4)/11.6 mM glucose] were subjected to 0.045 mN of resting tension, which produces a maximal contractile response to 87 mM K^+. Tissues were stimulated three times with K^+-H-T solution [54.4 mM NaCl/87 mM KCl/1.2 mM $MgCl_2$/2.5 mM $CaCl_2$/11.6 mM Hepes (pH 7.4)/11.6 mM glucose]. Where applicable, following relaxation in H-T solution, tissues were pre-incubated with inhibitors for 10 min. Tissues were stimulated with U-46619 in the absence or continued presence of inhibitors in H-T solution. At selected times, tissues were rapidly frozen in 10 % (w/v) TCA (trichloroacetic acid)/10 mM DTT in dry ice/acetone, washed (3 × 1 ml) with dry ice-cold 10 mM DTT/acetone, and freeze-dried for 16 h. In some instances, inhibitors were added after contraction had reached steady-state.

Depletion of intracellular and extracellular Ca^{2+}

Tissues were stimulated three times with K^+-H-T solution, relaxed for 10 min in H-T solution, stimulated with U-46619 or caffeine in H-T solution, and the agonists washed out with H-T solution. When tension returned to baseline, tissues were incubated in Ca^{2+}-free H-T solution containing 2 mM EGTA for 5 min to remove extracellular Ca^{2+}. U-46619 or caffeine was then added in Ca^{2+}-free H-T solution containing 2 mM EGTA. Intracellular stores were depleted by 60 min exposure to Ca^{2+}-free H-T solution containing 2 mM EGTA in the presence or absence of CPA (cyclopiazonic acid) before application of U-46619 or caffeine. Controls for all experiments with de-endothelialized rat caudal arterial smooth muscle verified that buffer changes alone had no effect on contraction.

α-Toxin permeabilization

Helical strips of de-endothelialized rat caudal artery, mounted on a force transducer, were incubated for 40 min in H-T solution. Tissues were stimulated with K^+-H-T solution and relaxed in H-T solution three times, removed from the force transducer and placed in pCa 9 solution [20 mM Tes (pH 6.9)/4 mM K_2EGTA/5.83 mM $MgCl_2$/7.56 mM potassium propionate/3.9 mM Na_2-ATP/0.5 mM dithioerythritol/16.2 mM phosphocreatine/15 units/ml creatine kinase] for 5 min. Permeabilization was achieved by incubation for 30 min in 0.25 ml of pCa 6.5 solution containing 8000 units of α-toxin (Sigma). Tissue strips were then washed three times with pCa 9 solution, and remounted on the force transducer. Fixed [Ca^{2+}] solutions were prepared as described previously [28] with the exclusion of sodium azide and the addition of the Ca^{2+} ionophore A23187 (10 μM).

Quantification of LC_{20} phosphorylation levels

De-endothelialized rat caudal arterial smooth-muscle strips, untreated or stimulated with U-46619 (1 μM) in the absence or presence of SQ-29548 (1 μM) or Y-27632 (10 μM), were quick-frozen at selected times, and LC_{20} phosphorylation was analysed by urea/glycerol-PAGE and Western blotting with anti-LC_{20}, as described previously [29]. Western blots were analysed with Image Master 1D software.

Rhotekin binding assay for detection of activated GTP-RhoA

Tissues stimulated with 1 μM U46619 or 10 μM GTP[S] were quickly frozen by immersion in liquid N_2. Eight frozen helical strips from each treatment were collected, homogenized in 600 μl of lysis buffer [25 mM Hepes (pH 7.5)/150 mM NaCl/1 % (v/v) Igepal CA-630/10 mM $MgCl_2$/1 mM EDTA/10 % (v/v) glycerol/20 μg/ml leupeptin/0.2 mM Pefabloc SC®/1 μg/ml aprotinin/1 μg/ml pepstatin A], incubated with end-over-end rotation for 30 min at 4 °C, and clarified by centrifugation at 14000 ***g*** for 10 min. Protein concentration was determined using the bicinchoninic acid method (Pierce), and supernatants containing 300 μg of total protein (in 500 μl) were incubated with 32.5 μg of Rhotekin Rho-binding domain-coupled agarose beads (Upstate USA, Inc.) at 4 °C for 45 min. Beads were washed twice with lysis buffer, and suspended in 40 μl of SDS gel sample buffer [30]. The total amount of RhoA, as well as the amount of RhoA that did not bind to the beads, was assessed by subjecting 25 μl samples, taken before and after pull-down, to Western blotting. Samples were resolved by SDS/PAGE (15 % gels) and the proteins were transferred to a 0.2 μm nitrocellulose membrane (Bio-Rad). After blocking with 0.5 % I-Block (Tropix, Bedford, MA, U.S.A.), the membranes were incubated with anti-RhoA (1:500 dilution) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, U.S.A.), and the enhanced chemiluminescence signal was developed using West Femto reagent (Pierce).

Western blotting of CPI-17 and MYPT-1

Protein was extracted from freeze-dried tissues by addition of 200 μl of 50 mM Tris/HCl, pH 6.8, containing 1 % SDS and 100 μM di-isopropylfluorophosphate for MYPT1, or 50 mM Tris/HCl, pH 6.8, containing 1 % SDS and 15 % glycerol for CPI-17. Samples were heated to 95 °C for 5 min, and then mixed for 60 min before the addition of 100 μl of 2× sample buffer prior

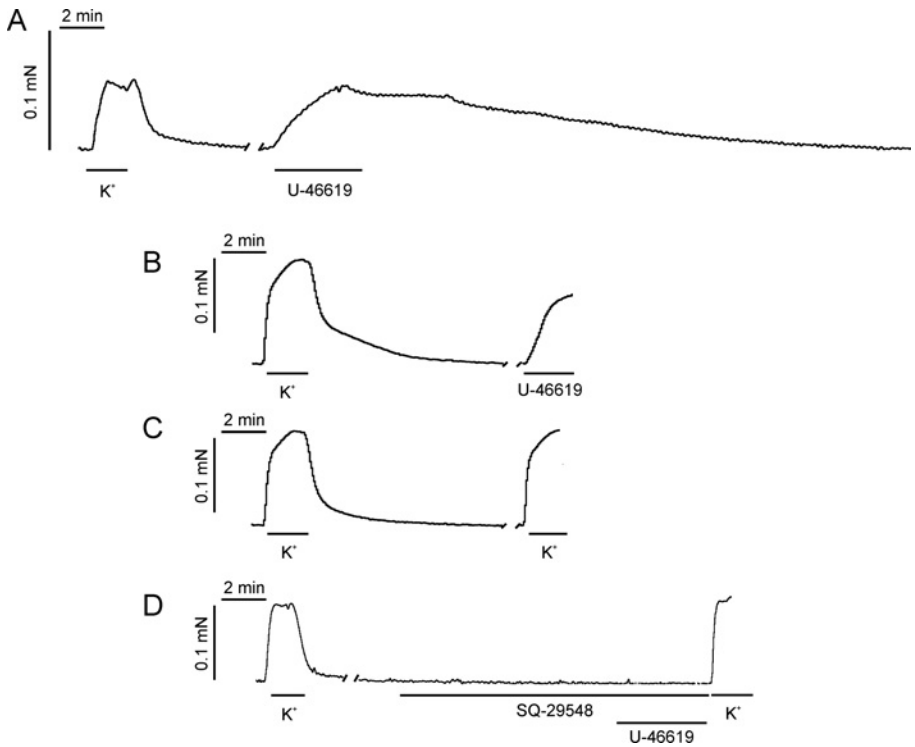

Figure 1 U-46619 elicits rat caudal arterial smooth muscle contraction via TP receptor activation

(**A**) A typical U-46619-induced contractile response of rat caudal arterial smooth muscle. De-endothelialized rat caudal arterial smooth muscle helical strips were contracted with 87 mM KCl (K^+) and relaxed in H-T solution. Subsequent addition of a submaximal concentration of U-46619 (0.1 μM) elicited a slow, sustained contractile response. Washout of the agonist resulted in slow relaxation. (**B**, **C**) The rate of contraction in response to a maximal concentration (1 μM) of U-46619 (**B**) was significantly lower than that due to K^+ depolarization (**C**). (**D**) U-46619 (1 μM)-mediated contraction was blocked by pre-treatment with the TP receptor antagonist SQ-29548 (1 μM). The viability of the preparation after treatment with SQ-29548 and U-46619 was verified by a normal contractile response to K^+ stimulation. ($n = 4$).

to SDS/PAGE at 35 mA for 3.5 h. For CPI-17, the gel was cut in half at the 68 kDa marker, and the lower-molecular-mass proteins were transferred to a 0.2 μm PVDF membrane on a Mini Protean II (Bio-Rad) at 30 mA for 5 h in 10 mM Caps, pH 11.0/10 % methanol. For MYPT1, the higher-molecular-mass proteins were transferred to 0.2 μm nitrocellulose at 100 V for 30 min in 25 mM Tris/HCl containing 192 mM glycine, 1 % SDS and 20 % methanol. The blots were blocked with 5 % non-fat dried milk in TBST solution for 1 h, and incubated with 1 % non-fat dried milk/TBST containing either anti-[PThr38]-CPI-17 (1:3000 dilution) or anti-CPI-17 (1:10000 dilution) for 1 h. Membranes were washed and incubated with anti-rabbit IgG–horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Chemicon) before chemiluminescence signal detection and quantification, as described above. For anti-[PThr697]-MYPT1 and anti-[PThr855]-MYPT1 rabbit polyclonal antibodies, the secondary antibodies were anti-rabbit IgG-coupled to a 780 nm fluorochrome. For anti-MYPT1 (mouse monoclonal), an anti-mouse IgG coupled to a 680 nm fluorochrome was used as secondary antibody. Fluorochrome signals were detected and analysed using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB, U.S.A.)

Statistical analysis

Where applicable, values are presented as the means $\pm$ S.E.M., with n indicating the number of independent experiments for a given treatment. Statistical differences were determined using Student's t test, with $P < 0.05$ considered to be statistically significant; asterisks indicate statistically significant differences from control.

RESULTS

U-46619 elicits sustained contraction of rat caudal arterial smooth muscle via TP receptor activation

Preliminary experiments indicated that the TxA_2 mimetic U-46619 elicits contraction of de-endothelialized rat caudal arterial smooth muscle in a concentration-dependent manner, with half-maximal activation at approx. 50 nM and maximal contraction at 1 μM U-46619. Figure 1(A) depicts a typical contractile response to 0.1 μM U-46619. Following washout of U-46619, the tissue relaxed very slowly, similar to the response described previously for rabbit pulmonary artery [5], and probably reflecting a low dissociation rate of the ligand from the receptor or sustained inhibition of MLCP (see the Discussion). The response to a maximal concentration of U-46619 was slower than that elicited by K^+ depolarization (compare Figures 1B and 1C). The contractile response to U-46619 can be attributed exclusively to activation of TP receptors, since it was abolished by pre-incubation with the TP receptor antagonist SQ-29548 (1 μM), which did not affect the viability of the preparation, since a normal K^+-induced contraction followed removal of the TxA_2 mimetic and the TP receptor antagonist (Figure 1D).

U-46619-evoked contraction requires extracellular Ca^{2+} entry

The importance of Ca^{2+} entry in the mechanism of action of U-46619 was indicated by the effects of removal of extracellular Ca^{2+}. Following U-46619 (0.1 μM)-induced contraction and washout of the agonist, the tissue was exposed to Ca^{2+}-free solution for 5 min to remove extracellular Ca^{2+}. Addition of U-46619 (0.1 μM) then failed to elicit a contractile response

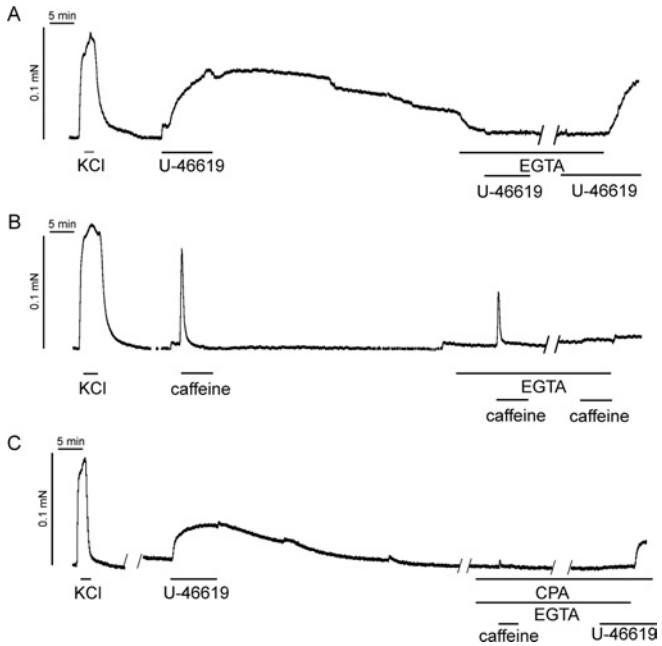

Figure 2 The effect of removal of extracellular Ca^{2+} on U-46619-mediated contraction of rat caudal arterial smooth muscle

(**A**) In the presence of extracellular Ca^{2+} (H-T solution), U-46619 (0.1 μM) elicited a typical contractile response, as seen in Figure 1(A). In the absence of extracellular Ca^{2+} (5 min exposure to H-T solution containing 2 mM EGTA), U-46619 (0.1 μM) failed to induce contraction. Following 60 min exposure to EGTA-containing solution, U-46619 again failed to elicit a contractile response. Replenishment of extracellular Ca^{2+} in the presence of U-46619 resulted in an immediate contractile response. ($n = 4$). (**B**) In control experiments, caffeine (20 mM) elicited a contractile response in the presence of extracellular Ca^{2+} and following removal of extracellular Ca^{2+}, but not following depletion of Ca^{2+} stores ($n = 4$). (**C**) Following K^+ (87 mM)- and U-46619 (0.1 μM)-induced contractions, tissue was exposed to EGTA and CPA (10 μM) prior to addition of caffeine (20 mM) for 5 min in the continued presence of EGTA and CPA. After prolonged exposure to EGTA-containing solution in the continued presence of CPA, addition of U-46619 (0.1 μM) had no effect until extracellular Ca^{2+} was provided ($n = 3$). Similar responses were observed when a higher concentration of U-46619 (1 μM) was used.

(Figure 2A), although caffeine (20 mM) did activate contraction under identical conditions (Figure 2B), verifying that the brief exposure to Ca^{2+}-free solution did not empty intracellular stores. These results suggest that U-46619 elicits contraction of rat caudal arterial smooth muscle by activating Ca^{2+} entry from the extracellular space, which may or may not involve Ca^{2+}-induced Ca^{2+} release from the SR (sarcoplasmic reticulum). If the tissue was subsequently exposed to EGTA-containing solution for a prolonged time period (60 min) to deplete intracellular Ca^{2+} stores, as verified by the loss of a contractile response to caffeine (Figure 2B), U-46619 again failed to elicit contraction (Figure 2A). Restoration of extracellular Ca^{2+} in the presence of U-46619 resulted in contraction (Figure 2A). These data indicate that U-46619-induced contraction utilizes Ca^{2+} from the extracellular Ca^{2+} pool, rather than the SR. The lack of importance of SR Ca^{2+} in the U-46619-induced contraction was verified by the inability of the SR Ca^{2+}-ATPase inhibitor CPA to block U-46619-induced contraction in the presence of extracellular Ca^{2+} (Figure 2C). Similar results were obtained with thapsigargin (results not shown).

U-46619-induced entry of extracellular Ca^{2+} occurs predominantly via nicardipine-sensitive Ca^{2+} channels

The voltage-gated Ca^{2+} channel antagonist nicardipine inhibited both U-46619 (1 μM)- and KCl (87 mM)-induced contractions

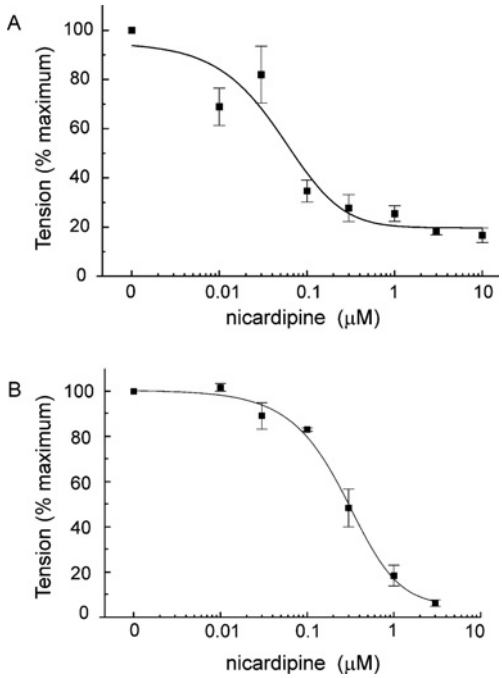

Figure 3 The effect of nicardipine on U-46619-induced contraction of rat caudal arterial smooth muscle

De-endothelialized smooth muscle strips were stimulated with U-46619 (1 μM) (**A**) or 87 mM KCl (**B**). Once steady-state tension development was complete, increasing concentrations of nicardipine were added, and relaxation of the tissue was monitored [$n = 3$–8 (**A**); $n = 4$ (**B**)].

in a concentration-dependent manner (Figure 3). The IC_{50} values were 0.1 μM for inhibition of U-46619-induced contraction and 0.3 μM for K^+-induced contraction. Maximal inhibition of contraction by nicardipine was approx. 80 % for U-46619-induced contraction (Figure 3A), in contrast with the complete inhibition of K^+-induced contraction (Figure 3B), suggesting that U-46619 also activates Ca^{2+} entry via a mechanism that is insensitive to nicardipine or triggers Ca^{2+} sensitization of contraction (see below). We confirmed that nicardipine did not affect Ca^{2+} release from intracellular stores, nor did it affect the functioning of the contractile machinery, since the contractile response to caffeine was maintained in the presence of nicardipine (results not shown).

U-46619-induced contraction involves increased phosphorylation of LC_{20} catalysed by MLCK

Figure 4(A) shows that LC_{20} are phosphorylated in response to U-46619 (1 μM), phosphorylation increasing from approx. 10 % in the resting tissue to approx. 25 % in response to the TxA_2 mimetic. This increase in LC_{20} phosphorylation would account for the contractile response to the same concentration of U-46619 observed in Figure 1(B). U-46619-induced LC_{20} phosphorylation was prevented by pre-incubation with the TP receptor antagonist SQ-29548 (1 μM) or the ROK inhibitor Y-27632 (10 μM) (Figure 4A). Consistent with the importance of LC_{20} phosphorylation in U-46619-induced contraction of rat caudal arterial smooth muscle, pre-treatment with the MLCK inhibitor ML-7 (30 μM) virtually abolished the contractile response to the TxA_2 mimetic (compare Figures 4B and 4C): the steady-state U-46619-induced force was 0.043 ± 0.002 mN ($n = 3$) in the absence, and 0.007 ± 0.001 mN ($n = 3$) in the presence, of ML-7. Furthermore, addition of ML-7 (30 μM) to tissues pre-contracted with U-46619

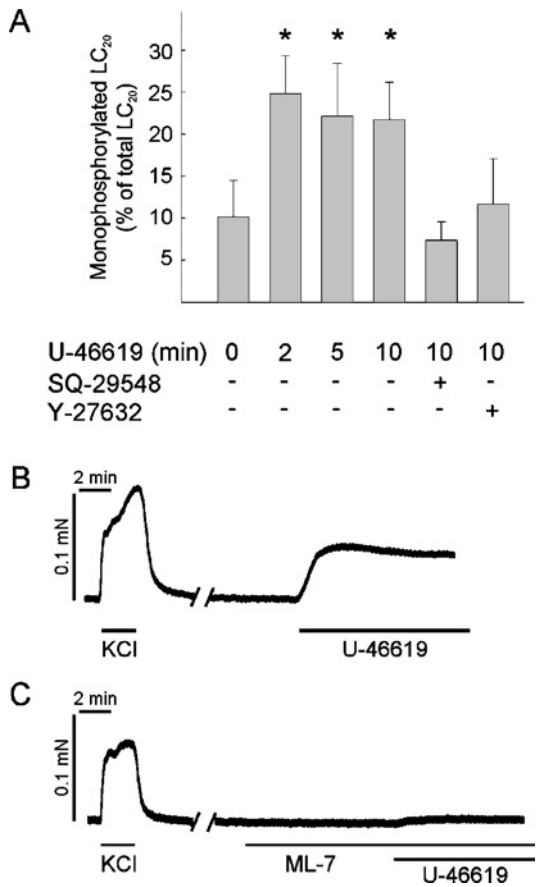

Figure 4 U-46619-induced contraction of rat caudal arterial smooth muscle correlates with phosphorylation of LC_{20} by MLCK

(A) De-endothelialized smooth muscle strips were untreated, stimulated with U-46619 (1 μM) for 2, 5 or 10 min, with U-46619 (1 μM) in the presence of SQ-29548 (1 μM) for 10 min, or with U-46619 (1 μM) in the presence of Y-27632 (10 μM) for 10 min. Phosphorylated and unphosphorylated LC_{20} were separated by urea/glycerol gel electrophoresis, detected by Western blotting with anti-LC_{20} and quantified by scanning densitometry ($n = 7$). Asterisks indicate statistically significant differences from control ($P < 0.05$). (**B**, **C**) Following treatment with KCl, de-endothelialized rat caudal arterial smooth muscle strips were pre-treated without (**B**) or with (**C**) ML-7 (30 μM) prior to addition of U-46619 (1 μM) ($n = 3$).

resulted in 85 % relaxation (results not shown). Similar results were obtained with two other MLCK inhibitors: ML-9 (300 μM) and wortmannin (1 μM) (results not shown).

U-46619 elicits Ca^{2+} sensitization in α-toxin-permeabilized rat caudal arterial smooth muscle

We then tested the hypothesis that the TxA_2 mimetic U-46619 may act via a Ca^{2+}-sensitization mechanism using α-toxin-permeabilized, Ca^{2+}-ionophore-treated rat caudal arterial smooth-muscle strips. Addition of GTP (10 μM) at a fixed [Ca^{2+}] (pCa 6.25) elicited a small contractile response (Figure 5A). Subsequent addition of U-46619 (1 μM) in the presence of GTP resulted in a marked increase in developed tension, i.e. Ca^{2+} sensitization (Figures 5A and 5C). Tension declined slightly over a 10 min incubation period under these conditions. However, if the bath solution (pCa 6.25; 10 μM GTP and 1 μM U46619) was replenished at 2 min intervals, the developed tension was sustained, suggesting that the slow decline in force observed in Figure 5(A) under these conditions was due to GTP hydrolysis. Furthermore, if GTP was not included in the bath, there was a much smaller contractile response to U-46619 (Figure 5C). Relaxation to resting tension oc-

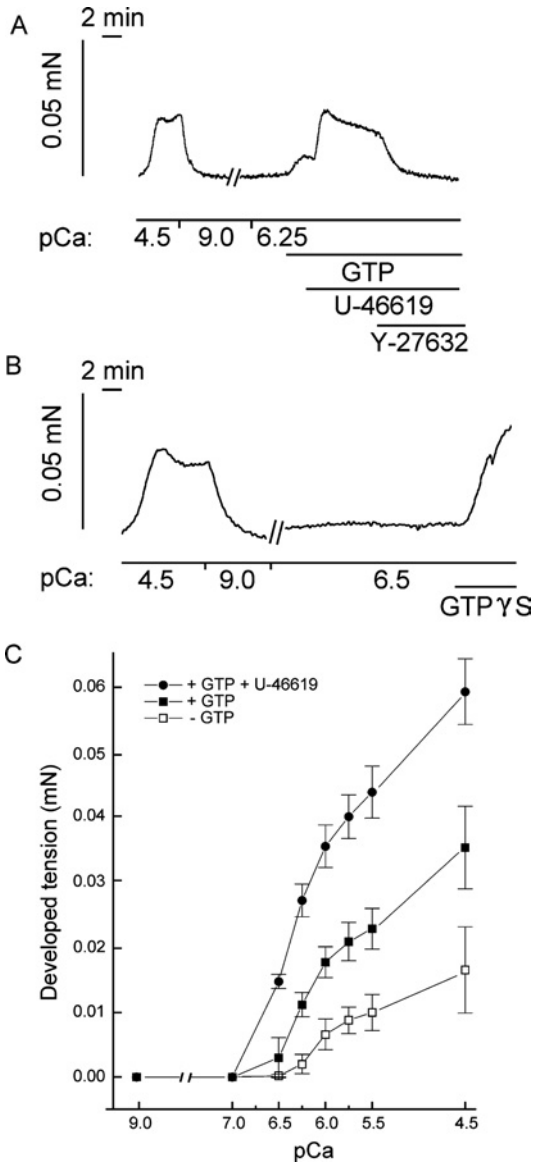

Figure 5 U-46619 induces Ca^{2+} sensitization in rat caudal arterial smooth muscle

(A) Following a control contraction–relaxation cycle, α-toxin-permeabilized, Ca^{2+}-ionophore (A23187)-treated, de-endothelialized smooth-muscle strips were exposed to threshold [Ca^{2+}] (pCa 6.25). Addition of GTP (10 μM) elicited a small contractile response. Subsequent addition of U-46619 (1 μM) induced a further increase in force. The contraction was fully reversed by Y-27632 (10 μM) treatment. (**B**) Following a control contraction–relaxation cycle, α-toxin-permeabilized tissue was transferred to sub-threshold [Ca^{2+}] (pCa 6.5). Addition of GTP[S] (10 μM) resulted in a robust Ca^{2+}-sensitization response ($n = 4$). (**C**) Cumulative data showing the Ca^{2+} sensitivity of contraction of α-toxin-permeabilized strips in the absence and presence of GTP (10 μM) and U-46619 (1 μM).

curred on addition of the ROK inhibitors Y-27632 (10 μM) (Figure 5A) or H-1152 (results not shown). The effect of U-46619 at fixed [Ca^{2+}] is similar to the typical Ca^{2+}-sensitization response exemplified by the addition of the non-hydrolysable GTP analogue GTP[S] (10 μM) at pCa 6.5 (Figure 5B). The data presented in Figure 5(C) indicate that the U-46619-induced contractile response of α-toxin-permeabilized, Ca^{2+}-ionophore-treated tissue, in addition to involving Ca^{2+} sensitization, is absolutely dependent on Ca^{2+}, consistent with the critical requirement for Ca^{2+}/calmodulin-dependent MLCK activity.

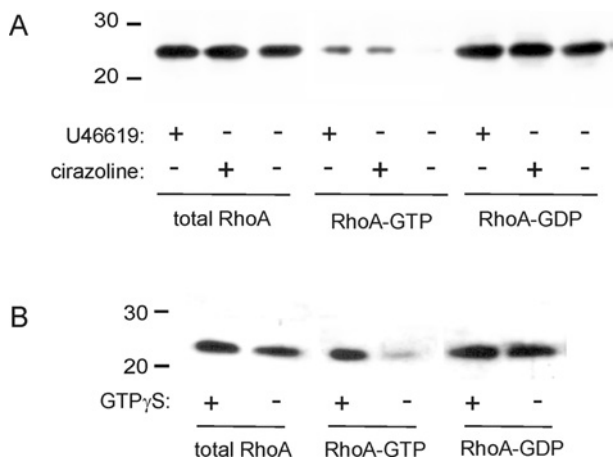

Figure 6 Activation of RhoA in response to U-46619 treatment of rat caudal arterial smooth muscle

(**A**) De-endothelialized smooth muscle strips were treated for 5 min with U-46619 (1 μM), the α_1-adrenoceptor agonist cirazoline (1 μM) or vehicle, and quick-frozen in liquid N_2. (**B**) α-Toxin-permeabilized, de-endothelialized smooth-muscle strips were treated with GTP[S] (10 μM) or vehicle and quick-frozen in liquid N_2. Tissues were homogenized (see the Experimental section), and samples were removed and added to SDS-gel sample buffer for analysis of total RhoA content. Activated (GTP-bound) RhoA was selectively pulled down from the remaining samples by interaction with the Rho-binding domain of Rhotekin coupled to agarose beads. Samples of washed beads (activated RhoA) and supernatants (inactive, GDP-bound RhoA) were treated with SDS-gel sample buffer. Proteins were separated by SDS/PAGE and RhoA was detected by Western blotting with anti-RhoA at 1:1000 dilution ($n = 2$).

U-46619-induced contraction involves activation of RhoA and ROK, but not PKC

The activation of RhoA in response to U-46619 treatment was assessed using the Rhotekin binding assay [31]. The RhoA-binding domain of Rhotekin, coupled to agarose beads, was used to specifically pull down activated RhoA (RhoA-GTP) from rat caudal arterial smooth-muscle homogenates prepared after exposure to U-46619 (1 μM), the α_1-adrenoceptor agonist cirazoline (1 μM as a positive control) or vehicle. As shown in Figure 6(A), control (untreated) tissue contained no detectable activated RhoA, although prolonged exposure did reveal a low level of RhoA-GTP under basal conditions, in agreement with the results of Sakurada et al. [32] with rabbit aorta. Treatment with U-46619 or cirazoline induced the formation of RhoA-GTP (Figure 6A). The proportion of total RhoA that was activated in response to U-46619 or cirazoline was small, as observed previously with rabbit aortic smooth muscle [23,32]. For comparison, α-toxin-permeabilized rat caudal arterial smooth muscle treated with GTP[S] (Figure 6B), which elicits a robust Ca^{2+}-sensitization response (see Figure 5B), is included as another positive control to illustrate the GTP[S]-induced activation of RhoA.

Y-27632 is widely used as a selective inhibitor of ROK, but recent studies have suggested that it is also capable of inhibiting PKC, particularly PKCδ [21]. We verified the effects of Y-27632 at 10 μM, a concentration commonly used in intact cell and tissue preparations to inhibit ROK, on the ability of cPKC (a mixture of Ca^{2+}-dependent α, β and γ isoforms) and Ca^{2+}-independent PKCδ to phosphorylate CPI-17, a PKC substrate that has been implicated in Ca^{2+} sensitization of smooth-muscle contraction. Y-27632 had no significant effect on the rate of phosphorylation of CPI-17 by cPKC (27.1 μmol of P_i/min per mg of PKC). On the other hand, and confirming the work of Eto et al. [21], 10 μM Y-27632 had a marked inhibitory effect on PKCδ activity: the rate of phosphorylation of CPI-17 (2.0 μmol of P_i/min per mg of PKCδ) was reduced significantly (by 73 %; $P < 0.05$, $n = 3$) in the presence of Y-27632.

Since Y-27632 is an effective inhibitor of PKCδ with CPI-17 as substrate, it was important to use another PKC inhibitor in addition to Y-27632 in experiments with rat caudal arterial smooth muscle, to discriminate between effects of the kinase inhibitor on ROK and PKCδ. We also used H-1152, which has been shown to be a potent ROK inhibitor (K_i 1.6 nM) with little effect on PKC (K_i 9.3 μM) [33]. Figure 7 shows the effects of Y-27632, H-1152 (ROK inhibitors) and GF-109203x (a PKC inhibitor) on U-46619-induced contraction of the rat caudal artery. U-46619 (1 μM)-induced contraction (Figure 7A) was abolished by pre-treatment with 10 μM Y-27632 (Figure 7B) or 100 nM H-1152 (Figure 7C), but unaffected by pre-treatment with 5 μM GF-109203x (Figure 7D), indicating that ROK, but not PKC, is involved in the contractile response to activation of TP receptors in this tissue. Furthermore, if tissues were pre-contracted by exposure to U-46619 (1 μM), addition of Y-27632 (10 μM) induced relaxation in the continued presence of the TxA_2 mimetic (Figure 7E). As a positive control, treatment with the PKC-activating phorbol ester PdBu (phorbol 12,13-dibutyrate; 1 μM) elicited a sustained contractile response (Figure 7F), which was blocked by pre-treatment with the PKC inhibitor GF-109203x (100 nM) (Figure 7G). Cumulative data (Figure 7H) confirm the inhibition of U-46619-induced contraction by inhibition of ROK, but not PKC.

Does U-46619-induced contraction involve phosphorylation of CPI-17 and/or MYPT1?

Once we had established that ROK and not PKC was the kinase involved in U-46619-dependent Ca^{2+} sensitization, we investigated which ROK substrate, MYPT1 and/or CPI-17, was phosphorylated upon agonist stimulation. Phosphorylation of CPI-17 (at Thr-38) and of MYPT1 (at Thr-697 and Thr-855) in rat caudal arterial smooth muscle in response to U-46619 was examined using phosphospecific antibodies. In the case of CPI-17, as a positive control tissues were treated with phorbol ester (1 μM PdBu) to activate PKC. As shown in Figures 7(F) and 7(H), treatment with PdBu elicited sustained contractions that reached levels of force comparable with that elicited by U-46619. The Western blots in Figure 8(A) verify the specificity of anti-[PThr38]-CPI-17 for the phosphorylated protein, with a detection limit of 0.25 ng (Figure 8A, panel a) and recognition of both phosphorylated and unphosphorylated CPI-17 by anti-CPI-17 with a detection limit of 25 ng (Figure 8A, panel b). Tissues were quick-frozen in TCA/acetone at selected times after addition of 1 μM PdBu for analysis of CPI-17 phosphorylation by SDS/PAGE and Western blotting with anti-[PThr38]-CPI-17 (Figure 8B). No phosphorylated CPI-17 was detected prior to the addition of PdBu and, as expected, CPI-17 was phosphorylated at Thr-38 during contraction induced by the phorbol ester. These results are consistent with activation of PKC by PdBu, leading to CPI-17 phosphorylation and Ca^{2+} sensitization of contraction. A similar approach was used to assess the phosphorylation of CPI-17 in response to U-46619 (1 μM). Tissues were quick-frozen at selected times after U-46619 addition, and CPI-17 phosphorylation was examined as before. In contrast with the effect of PdBu, U-46619 failed to elicit an increase in CPI-17 phosphorylation (Figure 8C). CPI-17 phosphorylation was also not observed 0.25, 0.5 and 1 min after addition of U-46619 (results not shown).

To assess the phosphorylation status of MYPT1 at Thr-697 and Thr-855 in response to U-46619 treatment, we used phospho-specific polyclonal antibodies that recognize MYPT1 only when phosphorylated at Thr-697 or Thr-855. The specificity of

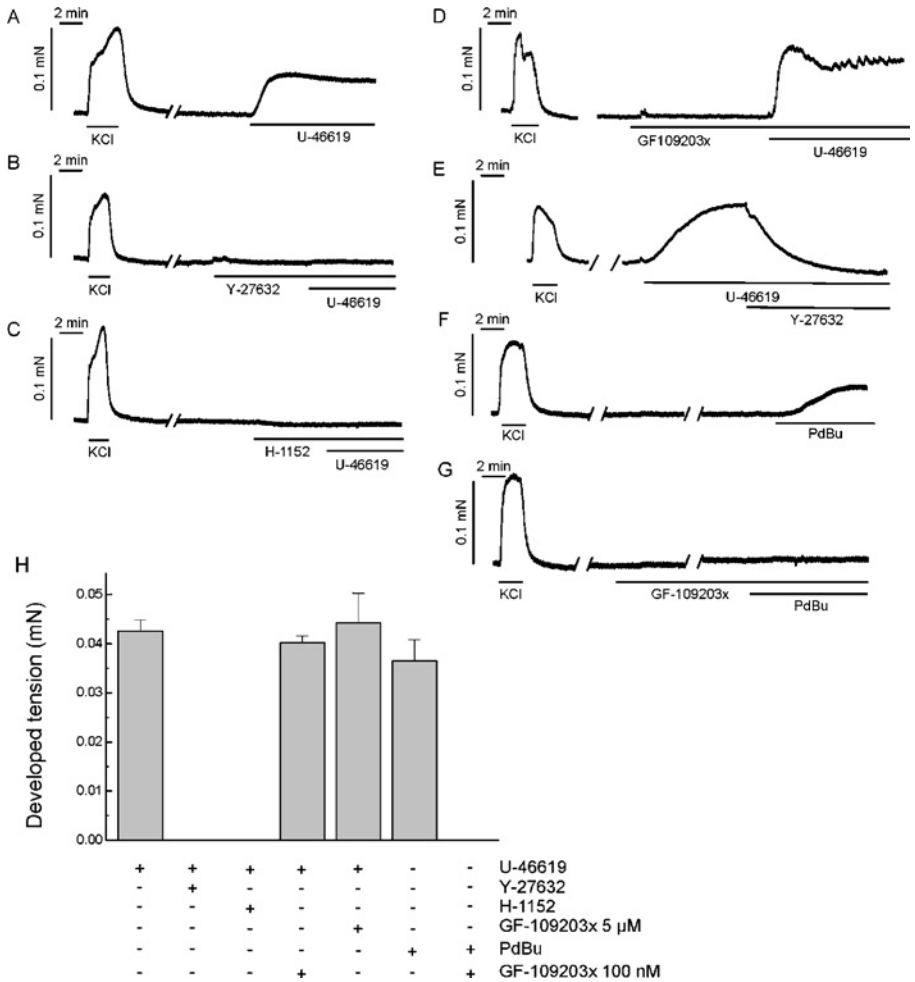

Figure 7 The effects of Y-27632, H-1152 and GF109203x on U-46619-induced contraction of rat caudal arterial smooth muscle

Following initial control K^+-induced contractions, tissues were either untreated (**A**) or pre-treated with Y-27632 (10 μM) (**B**), H-1152 (100 nM) (**C**) or GF109203x (5 μM) (**D**) prior to addition of U-46619 (1 μM). (**E**) Tissue pre-contracted with U-46619 (1 μM) was treated with Y-27632 (10 μM) in the continued presence of U-46619. Control tissues were treated with PdBu (1 μM) without (**F**) or with (**G**) pre-treatment with GF-109203x (0.1 μM). (**H**) Cumulative data ($n = 8$, except for 100 nM GF109203x and H-1552, in which cases $n = 4$).

anti-[PThr697]-MYPT1 was validated as shown in Figure 9(A): the antibody recognized phosphorylated, but not unphosphorylated, MYPT1 (Figure 9A, lanes 6 and 7). Untreated rat caudal arterial smooth muscle exhibited a basal level of MYPT1 Thr-697 phosphorylation (Figures 9A and 9B). Treatment with U-46619 (1 μM) or U-46619 and Y-27632 (10 μM) did not cause a significant change in phosphorylation at Thr-697 (Figures 9A and 9B). Basal phosphorylation was also detected at Thr-855 in unstimulated tissue, but in this case was significantly increased in response to U-46619 treatment (Figure 9C and 9D). Both the U-46619-induced increase in Thr-855 phosphorylation and the basal level of phosphorylation at this site were inhibited by Y-27632 (Figures 9C and 9D).

DISCUSSION

Figure 10 depicts our current understanding of the signal transduction pathway underlying U-46619-mediated contraction of rat caudal arterial smooth muscle based on the results described herein, with some steps inferred from earlier work with other smooth muscle, as well as non-muscle cell types. U-46619-induced contraction is mediated by the TP receptor, since it was completely blocked by the TP receptor antagonist SQ-29548 (Figure 1D). U-46619-induced contraction correlated with activation of RhoA (Figure 6A), most likely via coupling of the TP receptor to $G_{12/13}$ and activation of Rho-GEF via direct interaction of the G_α subunit with the GEF [12]. Activated GEF, in turn, activates RhoA via GTP/GDP exchange and dissociation from Rho-GDI (guanine nucleotide dissociation inhibitor) [10]. The downstream target of activated RhoA that is relevant to U-46619-induced Ca^{2+} sensitization appears to be ROK, since contraction was blocked in both intact and α-toxin-permeabilized, Ca^{2+}-ionophore-treated tissue by the ROK inhibitors Y-27632 and H-1152 (Figures 5A, 7B and 7C).

Two independent lines of evidence indicate that PKC is not involved in U-46619-induced contraction of the rat caudal artery. First, the PKC inhibitor GF109203x had no effect on U-46619-induced contraction (Figures 7D and 7H). Secondly, CPI-17 was not phosphorylated in response to U-46619 (Figure 8C). Although PKC involvement in U-46619-mediated contractile responses has been ruled out in this tissue, it is important to recognize that this may not be the case in other tissues. For example, Nobe and Paul [34] concluded that both PKC and ROK are involved in U-46619-induced contraction of porcine coronary arteries, a vascular

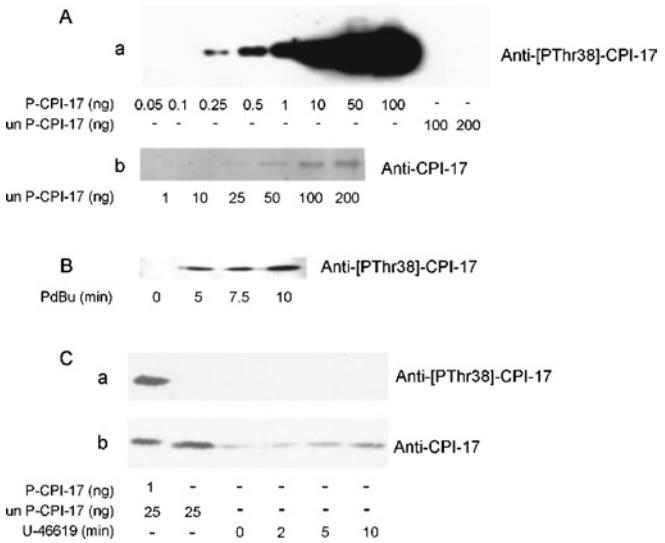

Figure 8 Analysis of CPI-17 phosphorylation in rat caudal arterial smooth muscle

(**A**) Determination of the detection limit of anti-[PThr38]-CPI-17 and anti-CPI-17. Different amounts of phosphorylated (P-CPI-17) or unphosphorylated recombinant CPI-17 (unP-CPI-17) were subjected to SDS/PAGE and Western blotting with anti-[PThr38]-CPI-17 (a) or anti-CPI-17 (b). (**B**) Phosphorylation of CPI-17 in response to PdBu treatment. Tissue samples were quick-frozen at the indicated times following addition of PdBu (1 μM), proteins were separated by SDS/PAGE, and CPI-17 phosphorylation at Thr-38 was assessed by Western blotting with anti-[PThr38]-CPI-17. (**C**) Lack of phosphorylation of CPI-17 in response to U-46619 treatment. Tissue samples were quick-frozen at selected times following addition of U-46619 (1 μM), proteins were separated by SDS/PAGE and CPI-17 phosphorylation at Thr-38 was assessed by Western blotting with anti-[PThr38]-CPI-17 (a). Blots were subsequently re-probed with anti-CPI-17, which recognizes both phosphorylated and unphosphorylated CPI-17 (b) ($n = 3$).

bed in which contraction is characterized by having two distinct components: a phasic component involving Ca^{2+} release from the SR and PKC-mediated Ca^{2+} sensitization, and a tonic component involving Ca^{2+} influx and ROK-mediated Ca^{2+} sensitization. With this in mind, it is important to consider that, although Y-27632 is reported to be a selective inhibitor of ROK, the present results, along with those of Eto et al. [21], clearly indicate that Y-27632 also inhibits PKCδ-catalysed phosphorylation of CPI-17 at a concentration (10 μM) that is effective in blocking Ca^{2+} sensitization (Figure 5A). Our results clearly demonstrate that the effects of Y-27632 were due to inhibition of ROK, and not PKC.

We had anticipated that MYPT1 would be phosphorylated by activated ROK, resulting in inhibition of MLCP and Ca^{2+} sensitization of contraction, but it was of particular importance to identify the sites of phosphorylation. Phosphorylation at Thr-697 inhibits MLCP activity [14]. The effect of phosphorylation at Thr-855 is unclear: studies with a C-terminal fragment of MYPT1 indicate that phosphorylation at this site causes dissociation from myosin [15], but the *N*-terminal domain of MYPT1 also contains binding sites for phosphorylated myosin and LC_{20} [35]. Although examples of phosphorylation of Thr-697 in intact smooth muscle in response to agonist stimulation have been provided [20,36–38], in the only other study in which phosphorylation of CPI-17 and MYPT1 in response to U-46619 treatment has been investigated (using cultured rat aortic smooth-muscle cells), small increases in phosphorylation of MYPT1 at Thr-697 and of CPI-17 at Thr-38 were observed [39]. However, in intact smooth muscle we observed no significant increase in phosphorylation of Thr-697 of MYPT1 (Figures 9A and 9B) or any phosphorylation of Thr-38 of CPI-17 (Figure 8C) in response to U-46619 treatment, although a phorbol ester was capable of inducing phosphorylation of CPI-17 (Figure 8B). We did, however, detect a significant increase in phosphorylation of MYPT1 at Thr-855 in response to U-46619 treatment (Figures 9C and 9D). We conclude, therefore, that ROK-mediated Ca^{2+} sensitization in rat caudal arterial smooth muscle may be mediated by Thr-855 phosphorylation of MYPT1. In addition, the finding that Y-27632 inhibits the basal level of Thr-855 phosphorylation (Figure 9D), but not of Thr-697 (Figure 9B), suggests that there is a basal level of ROK activity maintaining MLCP in a partially inhibited state in the resting muscle. The observations that Y-27632 did not reduce basal Thr-697 phosphorylation, and that U-46619 treatment did not increase phosphorylation at this site, suggest that Thr-697 is not a ROK phosphorylation site in this tissue. Phosphorylation of MYPT1 at Thr-855, but not Thr-697, has also been observed in rabbit portal vein and vas deferens in response to agonist stimulation, but in these cases CPI-17 phosphorylation at Thr-38 was also detected [21]. In chicken amnion smooth muscle, which lacks CPI-17, GTP[S]-induced Ca^{2+} sensitization correlated with an increase in phosphorylation of MYPT1 at Thr-855, but not Thr-697 [40]. Thr-697 phosphorylation was detected at rest in rabbit femoral arterial smooth muscle, but did not increase in response to agonist stimulation; Thr-855 phosphorylation was not investigated in this study [22].

A key step in the contractile response to U-46619 appears to be the entry of extracellular Ca^{2+}, since it was abolished by removal of extracellular Ca^{2+} (Figure 2A). The inhibitory effect of nicardipine (Figure 3A) on U-46619-induced contraction indicated that Ca^{2+} entry elicited by U-46619 occurs predominantly via voltage-gated Ca^{2+} channels. This leads us to speculate that ROK-mediated phosphorylation may act directly or indirectly to affect the gating of L-type Ca^{2+} channels. Furthermore, the fact that approx. 20 % of the U-46619-mediated contractile response was insensitive to nicardipine suggests two additional possibilities: (i) U-46619 stimulation may co-activate both nicardipine-sensitive and -insensitive Ca^{2+} channels; or (ii) the level of Ca^{2+} sensitization caused by U-46619 stimulation is sufficient to elicit a small amount of tone under conditions in which most, but not all, Ca^{2+} channels are blocked. The fact that K^+-induced contractile responses could be completely inhibited by nicardipine confirms the absolute requirement for Ca^{2+}, and suggests that U-46619 elicits a larger degree of Ca^{2+} sensitization than does KCl [41]. We considered the possibility that ROK may phosphorylate a sarcolemmal K^+ channel, inhibiting the channel and leading to membrane depolarization, which in turn activates the voltage-gated Ca^{2+} channel. Precedence for such a mechanism has come from recent work [42] demonstrating that UTP suppresses delayed rectifier K^+ currents (K_{DR}), and depolarizes rat cerebral arteries via activation of ROK. This mechanism, however, does not appear to operate in rat caudal artery depolarized with high external [KCl] (141 mM), conditions under which potential-dependent K_{DR} currents would be inactive (results not shown). Furthermore, U-46619-induced, ROK-mediated contraction was retained in α-toxin-permeabilized, Ca^{2+}-ionophore-treated tissue, in which ion-channel function is eliminated. Taken together, these data indicate that U-46619-mediated Ca^{2+} sensitization in the rat caudal artery is independent of ion channels responsible for regulating membrane potential. The simplest explanation for ROK-mediated activation of Ca^{2+} entry, therefore, would be direct phosphorylation of a subunit of the L-type Ca^{2+} channel or an associated regulatory protein. In support of this hypothesis, Shabir et al. [43] recently provided evidence that Ca^{2+} channels in rat ureteric smooth muscle may be targets of ROK. This potential mechanism will be the subject of future investigations.

U-46619-mediated ROK activation and MLCP inhibition alone is insufficient to elicit contraction, and requires some level of

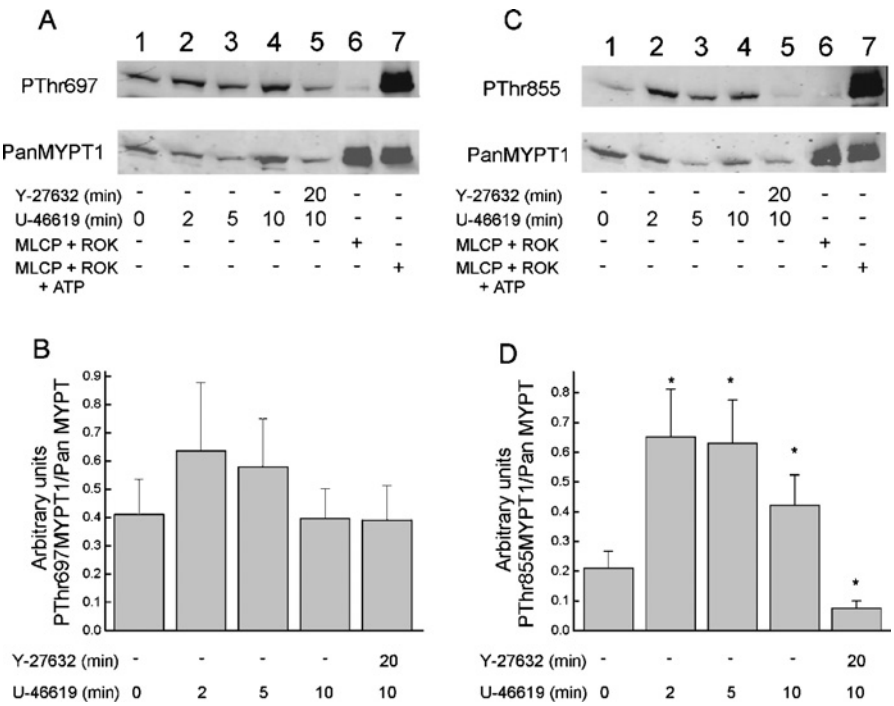

Figure 9 The effect of U-46619 treatment on MYPT1 phosphorylation at Thr-697 and Thr-855

Analysis of phosphorylation of MYPT1 in response to U-46619 treatment. Tissue samples were quick-frozen at selected times following addition of U-46619 (1 μM), proteins were separated by SDS/PAGE, and MYPT1 phosphorylation at Thr-697 and Thr-855 was assessed by dual labelling of Western blots with anti-[PThr697]-MYPT1 (rabbit polyclonal) and pan-MYPT1 (monoclonal) antibodies (**A**, **B**) or anti-[PThr855]-MYPT1 (rabbit polyclonal) and pan-MYPT1 (monoclonal) antibodies (**C**, **D**), all at 1:1000 dilution (see the Experimental section). To account for any variations in loading levels, the data are expressed in arbitrary units as the ratio of signal intensities of phosphorylated MYPT1:total MYPT1, the latter being detected by an antibody that recognizes both phosphorylated and unphosphorylated forms of the protein ($n = 8$). Phosphorylated MYPT1 was produced by incubating purified MLCP with ROK in the presence of ATP (MLCP + ROK + ATP). Unphosphorylated MYPT1 was from identical incubations in the absence of ATP (MLCP + ROK).

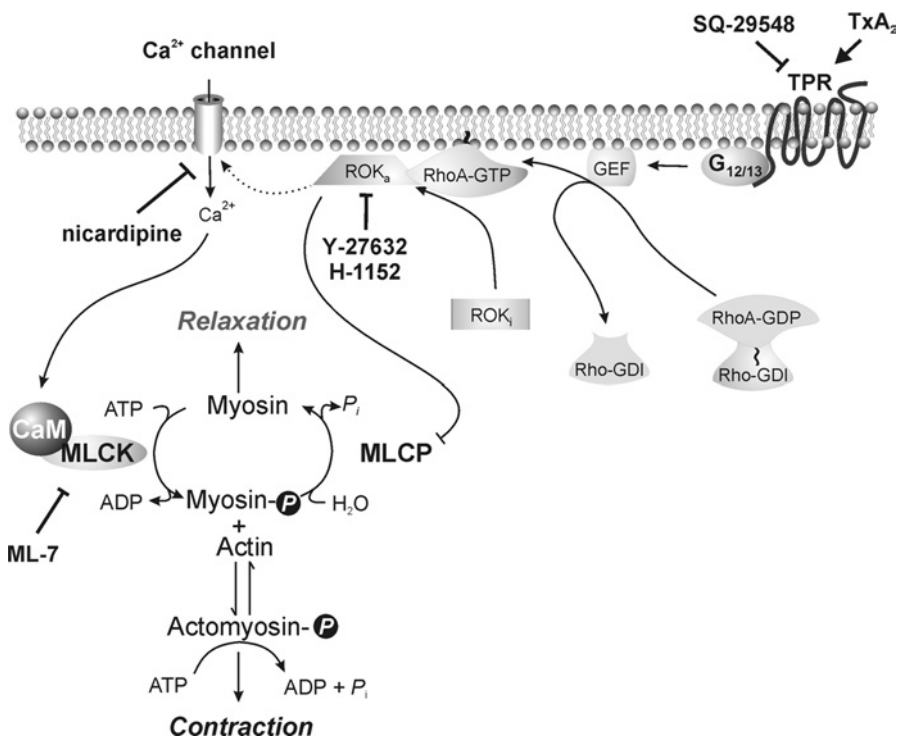

Figure 10 Proposed mechanism of U-46619-induced contraction of rat caudal arterial smooth muscle

Abbreviations undefined elsewhere: $G_{12/13}$ are heterotrimeric G-proteins; RhoA-GDP, inactive form of RhoA; RhoA-GTP, active form of RhoA; ROK_i, inactive form of ROK; ROK_a, activated form of ROK; CaM, calmodulin.

Ca^{2+}/calmodulin-dependent MLCK activation. Indeed, Ghisdal et al. [44] recently provided evidence for ROK-mediated activation of Ca^{2+} entry via non-selective cation channels in rat aorta and mesenteric arteries in response to noradrenaline. This mechanism appears to play a minor role in U-46619-induced contraction of the rat caudal artery, since this contractile response is retained in the presence of high extracellular [K^+] (141 mM; results not shown), at which the driving force for Ca^{2+} entry via non-selective cation channels would be very low or absent. Following U-46619-induced Ca^{2+} entry from the extracellular space, Ca^{2+} diffuses to the contractile machinery, where it binds to calmodulin to activate MLCK [28], increase the level of myosin regulatory light-chain phosphorylation, and activate cross-bridge cycling and contraction (Figure 10). A limited number of studies have investigated the involvement of myosin phosphorylation in U-46619-induced contraction, and have relied mostly on MLCK inhibitors. For example, Kaye et al. [45] demonstrated inhibition of the vasoconstrictor response to U-46619 in the cat pulmonary vascular bed by MLCK inhibition. Maeda et al. [46] demonstrated U-46619-induced Ca^{2+} sensitization of contraction of the bovine middle cerebral artery, a response that consisted of an LC_{20} phosphorylation-dependent and -independent component. Here, through direct quantification of LC_{20} phosphorylation levels, we have established a correlation between U-46619-evoked force and myosin phosphorylation (compare Figures 1B and 4A). Thus U-46619 elicited an increase in LC_{20} phosphorylation with a time course similar to that of force development. Furthermore, inhibition of U-46619-induced contraction by the TP receptor antagonist SQ-29548 or the ROK inhibitor Y-27632 also inhibited the increase in LC_{20} phosphorylation (Figure 4A). The observed LC_{20} phosphorylation can be attributed to MLCK, and not ZIP kinase (zipper-interacting protein kinase) or ILK (integrin-linked kinase), which are known to phosphorylate LC_{20} *in vitro*: (i) ZIP kinase [47] and ILK [48] phosphorylate LC_{20} at both Ser-19 and Thr-18, but we detected no diphosphorylation of LC_{20} in response to U-46619 treatment; (ii) U-46619-induced contraction of α-toxin-permeabilized rat caudal artery (Figure 5C), like contraction of the intact tissue (Figure 2A), is absolutely dependent on an increase in Ca^{2+} concentration, but ZIP kinase and ILK, unlike MLCK, are Ca^{2+} independent; and (iii) ML-7, ML-9 and wortmannin, used at concentrations that inhibit MLCK but not ZIP kinase or ILK, inhibited U-46619-induced contraction.

In the case of the rat caudal artery, there is no apparent rapid phasic component of the contractile response to U-46619 (in contrast with the typical biphasic response elicited by α_1-adrenoceptor activation [49]), and PKC inhibitors have no effect; nor is there any evidence of CPI-17 phosphorylation. In this tissue, as in some others, e.g. canine pulmonary venous smooth muscle [7] and rat pulmonary artery [8], there appears to be only the slow, sustained contractile response, which slowly relaxes (Figure 1A) due to slow agonist dissociation or sustained inhibition of MLCP with a reduced rate of LC_{20} dephosphorylation and relaxation. In the rat caudal artery we have provided evidence of RhoA activation, an increase in the phosphorylation of one known target of the ROK signalling cascade (MYPT1), but not of CPI-17, and inhibition of this response by a ROK inhibitor, but not by a PKC inhibitor. We conclude that ROK is activated and results in the phosphorylation of Thr-855 of MYPT1, but not Thr-38 of CPI-17 or Thr-697 of MYPT1. In the rat caudal artery, U-46619-mediated contractile responses have an absolute requirement for Ca^{2+}, which enters from the extracellular pool, is independent of intracellular Ca^{2+} stores and is blocked by ROK inhibition. Our results on the mechanism of activation of contraction of rat caudal arterial smooth muscle by U-46619 in comparison with related studies, using different vessels, emphasize the existence of tissue-specific TxA_2-mediated signalling pathways for regulation of vascular smooth-muscle contractility. Some of these tissue-specific differences may relate to differences in localization of TP receptors and their association with different proteins in signalling complexes, and to the relative degree to which TP receptors are coupled to $G_{12/13}$ in comparison with $G_{q/11}$ in the different smooth-muscle cell types.

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